ALKALINE pH, FREE SOLUTION CAPILLARY ELECTROPHORESIS METHOD

The present invention relates to a process for separating proteins and peptides by capillary electrophoresis using buffer system compositions comprising an additive for use in such separation.

Blood proteins are often analysed, in particular for diagnostic purposes. The detection of monoclonal proteins can allow early diagnosis, or it can allow therapies for certain diseases to be tracked.

Proteins are routinely separated by electrophoresis, either conventional gel electrophoresis, or by capillary electrophoresis (CE). One advantage of CE is the very small quantities of sample used for analysis. Further, the use of a capillary tube with a small internal diameter to carry out electrokinetic separation disperses the heat produced by the Joule effect extremely well. This highly efficient heat dissipation associated with the high electrical resistance of capillary tubes allows high tensions to be applied, and thus produces very short separation times. Free solution CE, in which the separation medium is a simple buffer solution, is of particular application as the capillary can readily be re-packed and filled with fresh solution between each analysis.

To improve the separation achieved using CE, one technique consists of using capillaries the internal surface of which has been treated. However, such coatings are not very stable and they deteriorate during use, and are thus of low reliability when carrying out a large number of analyses, which limits the advantage of the technique for routine analysis.

To analyse blood proteins using free solution CE, there is an advantage in using a buffer system with a pH of the order of 9 to 11, preferably about 10.

Alkaline buffer systems include borate buffers such as those described in United States patent US-A-5 120 413. Such buffers form complexes with glycoproteins. Most blood proteins are glycosylated. The formation of such complexes modifies the electrophoretic mobility of glycoproteins. With such a borate buffer, at a pH of about 10, blood proteins are usually divided into 6 fractions (gamma, beta-2, beta-1, alpha-2, alpha-1, albumin). There is a risk that some

monoclonal proteins will co-migrate with normal protein fractions, and during analysis, certain normal protein fractions may mask certain monoclonal proteins.

As an example, analysing serums containing monoclonal proteins, in particular certain proteins of the IgM kappa type, shows that the corresponding peak co-migrates with one of the protein fractions (the beta-2 fraction), resulting in a high risk of non detection of such IgM kappa.

The Applicant has now demonstrated that rapid and efficient protein separation can be carried out using free solution CE employing an alkaline buffer as the buffer system, i.e., with a pH of 9 to 11, more precisely about 10, comprising a zwitterionic biological buffer as the buffer and in addition, at least one additive that can increase the ionic strength of the buffer system.

The present invention provides an alkaline pH, free solution capillary electrophoresis method for analysing clinical samples comprising protein constituents, this method comprising at least one step in which the sample is introduced into a capillary tube containing a buffer system comprising, as the buffer, a biological buffer with a pKa at 25°C in the range 8.8 to 10.7 and at least one additive that can increase the ionic strength of the buffer system.

The Applicant has demonstrated that selecting a combination of a zwitterionic biological buffer with an alkaline pH of the order of 9 to 11, more precisely about 10, and an additive that can increase the ionic strength of the electrophoresis medium, can achieve improved separation.

The separations are reproducible. Further, the zwitterion has a majority of negative charges at a pH of about 10, which may be advantageous within the context of the invention.

Finally, as will become apparent from the examples, certain proteins appear and can be detected in the form of a peak that is more distinct or clearly separated from other fractions when compared with the detection achieved using a borate buffer.

As will also become apparent in the examples, separations carried out using the buffers of the present invention can produce a separation that is equivalent to or identical to that which can

be observed with other techniques, in particular gel electrophoresis, and with a greater accuracy, reproducibility and resolution.

Other characteristics and advantages of the invention will become apparent from the following detailed description made with reference to the accompanying drawings.

Figures 1A and 1B show electropherograms obtained by capillary electrophoresis using buffer systems of the invention.

Figures 1C and 1D respectively show the gel and the densitometric profile of the same serum using gel electrophoresis.

Figure 2A shows an electropherogram of a serum with a monoclonal gammapathy, analysed by capillary electrophoresis using a buffer system of the invention.

Figures 2B and 2C respectively show the gel and the denistometric profile of the same serum obtained with gel electrophoresis.

Figure 3A shows an electropherogram of a serum with biclonal gammapathy, analysed by capillary electrophoresis using a buffer of the invention.

Figures 3B and 3C respectively show the gel and densitometric profile of the same serum carried out by gel electrophoresis.

Figure 4A shows an electropherogram of a serum with low monoclonal gammapathy analysed by capillary electrophoresis using a buffer of the invention.

Figures 4B and 4C respectively show the gel and the densitometric profile of the same serum carried out by gel electrophoresis.

Figure 5A shows an electropherogram of a serum comprising a monoclonal protein migrating at the limit of the γ and β fractions, analysed by capillary electrophoresis using a buffer of the invention.

Figures 5B and 5C respectively show the gel and the densitometric profile of the same serum carried out by gel electrophoresis.

Figure 5D shows an electropherogram of the same serum analysed by capillary electrophoresis using the usual borate buffer.

Figure 6 shows ten electropherograms, numbered 1 to 10, of the same serum obtained during 10 successive analyses using capillary electrophoresis employing a buffer of the invention.

In accordance with the present invention, the buffer system can be any normal known buffer known as a biological buffer and having a pKa at 25°C in the range 8.8 to 10.7, i.e., compatible with *in vivo* applications, adapted to the desired separation, and useful for electrophoresis in general, and in particular for capillary electrophoresis. Preferably, biological buffers with a high buffering power with a pH of about 10 are selected.

Amongst the biological buffers useful according to the invention, a buffer of the Good type is particularly cited as the CAPS defined herebelow and analogs. The Good type buffers according to the inventionare zwitterionic and have a pKa at 25°C between 8,8 and 10,7. The buffer of the Good type and their analogs comprise amine and acid functions.

Particularly biological buffers suitable according to the invention that can be cited are AMPD (2-amino-2-methyl-1,3-propanediol), TABS (N-tris[hydroxymethyl]methyl-4-aminobutanesulphonic acid), AMPSO (3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulphonic acid), CHES (2-(N-cyclohexylamino)ethanesulphonic acid), CAPSO (3-[cyclohexylamino]-2-hydroxy-1-propanesulphonic acid), AMP (2-amino-2-methyl-1-propanol), CAPS (3-cyclohexylamino-1-propanesulphonic acid) and CABS (4-[cyclohexylamino]-1-butanesulphonic acid) and mixtures thereof. Other zwitterionic biological buffers can be used in the invention. The amino acid buffers are however not intended as a buffer or additive according to the present invention.

In accordance with the invention, AMPD, TABS, AMPSO, CAPSO, AMP, CAPS and CABS buffers are preferred. More preferably, CAPS, CAPSO or CABS are used. More particularly preferably, CAPS is used.

Compounds that can be cited for use as the additive to the buffer for use in accordance with the invention that can increase the ionic strength of the electrolyte are selected from alkali metal chlorides, sulphates, sulphonates, carbonates, carboxylates, fluorides and phosphates and mixtures thereof. Of these, alkali metal chlorides, sulphates and sulphonates and mixtures thereof are preferred.

More preferably, the sulphate is used.

Preferably, sodium or potassium salts are selected.

Of the additives cited above, sodium sulphate is preferred.

Preferably, in accordance with the invention, CAPS s associated with sodium sulphate.

These compounds are known *per se* and are commercially available.

The term "sample in accordance with the invention" means the biological sample to be analysed, diluted with a suitable diluting solution or buffer system, for example, or pure, which is analysed with the buffer system, i.e., the electrolyte, for example by introducing the sample into a capillary filled with that buffer.

The clinical sample for analysis and the term "clinical sample" as used here means any biological liquid from healthy humans or human patients. The human biological liquids can be normal or diseased serum, and also haemolysed serum, plasma, urine, or cerebro-spinal fluid. The processes and compositions according to the present invention are particularly useful for the analysis of serum, plasma, urine, or cerebro-spinal fluid.

The samples can also be synthetic proteins, and the method of the invention can, for example, be intended for production control.

The method of the invention is of particular application in analysing serum, and for separating blood proteins.

In blood samples, the blood proteins to be separated are primarily albumin and the α_1; α_2; β (or β_1 and β_2); and γ globulin fractions.

The pH of the buffer of the invention, i.e., the pH of the biological buffer with the additive, can be between 9 and 11, particularly preferably about 10.

The buffer systems of the invention can also comprise at least one pH-modifying component. The pH-modifying compound can be a compound selected from lithium hydroxide, sodium hydroxide, potassium hydroxide, rubidium hydroxide, caesium hydroxide, francium hydroxide, or a mono-, di-, tri- or tetra-alkyl ammonium hydroxide containing 1 to 8 carbon atoms in the alkyl portion.

In accordance with the invention, the biological buffers are used under the usual conditions, at concentrations in the buffer system of the order of 10 to 500 mM, preferably more than 20 and less than 200 mM.

The salts used as additives in accordance with the invention are used at concentrations in the buffer system of 10 mM to 500 mM, preferably 50 to 200 mM, more preferably about 150 mM.

The buffer systems of the invention can also comprise at least one additive comprising a negatively charged anionic pole at a pH of more than 9, and a hydrophobic portion, in particular C_6 to C_{22} alkyl-mono-, di- or tri- sulphonates, C_6 to C_{22} alkylmono-, di- or tri-carboxylates, C_6 to C_{22} alkylcarboxysulphonates, and in particular C_6 to C_{10} alkylsulphonates.

The above di- and tri-carboxylates, di- and tri-sulphonates and carboxysulphonates are thus combinations of one or more carboxylate or sulphonate functions on C_6 to C_{22} alkyl chains. Non limitative examples thereof are the 1,2,3-nonadecanetricarboxylic acid (three carboxylate functions and a en C_{19} alkyl chain), the 2-methyl-2-sulfooctadecanoic acid (one carboxylate function and one sulfonate function and a C_{18} alkyl chain) and the 1,12-dodecanedicarboxylic acid (two carboxylate functions and a C_{12} alkyl chain).

Preferably, octanesulphonate is used, in concentrations of the order of 1 to 5 mM, preferably 1 to 4 mM and more preferably 2.5 mM.

The buffer compositions of the invention are prepared in a manner that is normal when preparing buffer system compositions, namely by adding the constituents in the liquid form, or as a solid to be diluted, to an acceptable support. Usually, the support is water, either distilled or demineralised.

The materials used for the capillaries are those routinely employed in capillary electrophoresis. It is possible to use fused silica capillaries with an internal diameter of 5 to 200 μm. Preferably, capillaries with an internal diameter of less than 100 μm are used; more preferably, less than 50 μm. Preferably, capillaries with an untreated internal surface are used. The skilled person will be capable of adapting the nature and size of the capillary to the analytical requirements.

EXAMPLES

MATERIALS AND METHODS

A) Capillary electrophoresis (method A)

Capillary electrophoresis was carried out on clinical samples using a CE apparatus provided with a fused silica capillary with an internal diameter of 25 microns. Detection was carried out at 200 nm. The samples were placed in the apparatus's sample changer and automatically injected by hydrodynamic injection (50 mbars for 7 s). The samples were separated within 10 minutes by applying an electrical field of about 400 V/cm. The capillary was washed with 0.5 M sodium hydroxide before each analysis, then with the buffer system.

Buffer systems:

Analytical grade chemical substances were used.

A first buffer in accordance with the invention was prepared by dissolving 11.07 g of CAPS (molar mass 221.3 g/mole) and 21.3 g of sodium sulphate (molar mass 142.04 g/mole) in 1 litre (l) of demineralised water. The final concentration was 50 mM of CAPS and 150 mM of

sodium sulphate, and the pH was adjusted to 10.0 by adding sodium hydroxide pellets (molar mass: 40.0 g/mole).

A second, preferred, buffer system was prepared as described above, adding octanesulphonate in a concentration of 2.5 mM.

Electrophoresis carried out using method A above with the CAPS/sodium sulphate buffer produced a protein profile with 5 fractions, the gamma, beta, alpha-2, alpha-1 and albumin fractions, reading from left to right.

The borate buffer was prepared by dissolving 9.3 g of boric acid (molar mass: 61.83 g/mole) in 1 l of demineralised water, and 5.1 g of sodium hydroxide (molar mass: 40.0 g/mole). The final concentration was 150 mM and the pH was 10.0.

Electrophoresis carried out using method A above with the borate buffer produced a protein profile with 6 fractions, the gamma, beta-2, beta-1, alpha-2, alpha-1 and albumin fractions, reading from left to right.

B) Agarose gel electrophoresis (method B)

Agarose gel was used to carry out a comparative analysis of the blood proteins. 10 µl of serum was loaded into each well in the membrane applicator described in European patent EP-A-0 493 996, US-A-5 464 515 and US-A-5 405 516. The loaded applicator was then applied to the surface of an agarose gel for 30 seconds. The samples applied to this gel were separated by electrophoresis for about 7.5 minutes at a power of 20 W, using an instrument that could regulate the temperature to 20°C. After migration, the gel was dried and stained with acid black. After staining, the gel was decolorised and dried again. The gels were then analysed by densitometry to produce the protein profiles.

For a normal serum, a protein profile was obtained with 5 fractions, the gamma, beta, alpha-2, alpha-1 and albumin fractions, reading from left to right.

C) Clinical samples:

For the CE, the human serum was diluted to $1/10^{th}$ in the buffer system.

EXAMPLE 1

With the first and second buffers described above, normal serum was analysed.

Electrophoresis was carried out using method A above.

As can be seen from Figures 1A and 1B, the electropherograms obtained exhibited five peaks, successively attributed to γ, β, α_2, α_1 globulin and albumin , reading from left to right.

EXAMPLE 2 (comparative)

The protein profile (the gel in Figure 1C and its densitometric profile of Figure 1D) was obtained by analysing the same serum as in the preceding example using method B above. As can be seen from these figures, the protein profile obtained exhibited 5 fractions, γ, β, α_2, α_1 and albumin, reading from left to right. Comparison with the result obtained in Example 1 shows that the implementations of the invention can produce a protein profile with 5 fractions comparable with that obtained with agarose gel.

EXAMPLE 3

The second buffer described above was used to analyse a serum with monoclonal gammapathy.

The electrophoresis was carried out as described in Example 1.

As can be seen in Figure 2A, the electropherogram obtained exhibits five successive peaks, attributed to γ, β, α_2, α_1 globulin and albumin fractions respectively. Note the presence of a supplemental peak in the gamma fraction, corresponding to the monoclonal protein present in the analysed serum.

EXAMPLE 4 (comparative)

The protein profile (the gel in Figure 2B and its densitometric profile of Figure 2C) was obtained by analysing the same serum as in the preceding example using method B above. Comparison with the result obtained in Example 3 shows that the implementations of the invention can achieve a resolution equivalent to that obtained with agarose gel.

EXAMPLE 5

The second buffer described above was used to analyse a serum with biclonal gammapathy.

The electrophoresis was carried out as described in Example 1.

As can be seen in Figure 3A, the electropherogram obtained showed two supplemental peaks in the gamma fraction, corresponding to the two monoclonal proteins present in the analysed serum.

EXAMPLE 6 (comparative)

The protein profile (the gel in Figure 3B and its densitometric profile of Figure 3C) was obtained by analysing the same serum as in the preceding example using method B above. Comparison with the result obtained in Example 5 showed that the implementation of the invention can produce a resolution that is higher than the resolution obtained with an agarose gel. On agarose gel, one of the monocolonal protein co-migrated with the beta fraction.

EXAMPLE 7

The second buffer described above was used to analyse a serum with weak monoclonal gammapathy.

The electrophoresis was carried out as described in Example 1.

As can be seen in Figure 4A, the electropherogram obtained showed a small supplemental peak in the gamma fraction, corresponding to the monoclonal protein present in the analysed serum.

EXAMPLE 8 (comparative)

The protein profile (the gel in Figure 4B and its densitometric profile of Figure 4C) was obtained by analysing the same serum as in the preceding example using method B above. Comparison with the result obtained in Example 7 showed that the implementation of the invention can achieve a sensitivity substantially identical to that obtained with agarose gel.

EXAMPLE 9

The second buffer described above was used to analyse a serum with a monoclonal protein of the IgM kappa type migrating to the limit of the γ and β fractions.

As can be seen in Figure 5A, the electropherogram obtained showed a supplemental peak in the gamma fraction, corresponding to the monoclonal protein present in the analysed serum.

EXAMPLE 10 (comparative)

The protein profile (the gel in Figure 5B and its densitometric profile of Figure 5C) was obtained by analysing the same serum as in the preceding example using method B above. Comparison with the result obtained in Example 9 showed that the implementation of the invention can achieve a detection that is substantially identical to that obtained with agarose gel.

EXAMPLE 11 (comparative)

The procedure of Example 1 was followed, the buffer system used being the normal borate buffer prepared as indicated above.

Electrophoresis was carried out using method A above.

As can be seen in Figure 5D, the electropherogram obtained exhibited six successive peaks, attributed respectively to the γ, β_2, β_1, α_2, α_1 globulin and albumin fractions, reading from left to right.

No perturbation in the protein profile was observed with this borate buffer, only an increase in the percentage of the beta fraction above normal values that could give rise to suspecting the presence of a monoclonal protein in this serum; comparison with the result obtained in Example 9 shows that this implementation of the invention can achieve a higher resolution compared with that obtained with CE using the normal borate buffer for certain kappa IgM type monoclonal proteins.

EXAMPLE 12

Using the second preferred buffer, 10 consecutive analyses were carried out on the same serum by capillary electrophoresis using method A described above; as can be seen from Figure 6, the reproducibility of the profiles was excellent.